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A natural M RNA reassortant arising from two species of plant- and insect-infecting bunyaviruses and comparison of its sequence and biological properties to parental species ☆

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ABSTRACT

Reassortment allows multicomponent viruses to exchange genome segments, a process well-documented in the vertebrate- and arthropod-infecting members of the family *Bunyaviridae* but not between distinct species of the plant- and insect-infecting members of the genus *Tospovirus*. Genome sequence comparisons of a virus causing severe tospovirus-like symptoms in Florida tomato with *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) demonstrated that reassortment has occurred, with the large (L) and small (S) RNAs coming from GRSV and the medium (M) RNA coming from TCSV (i.e. $L_GM_TS_G$). Neither parental genotype is known to occur in the U.S. suggesting that $L_GM_TS_G$ was introduced as a reassortant. $L_GM_TS_G$ was transmitted by western flower thrips (*Frankliniella occidentalis* [Pergande]), and was not able to overcome the *Sw5* resistance gene of tomato. Our demonstration of reassortment between GRSV and TCSV suggests caution in defining species within the family *Bunyaviridae* based on their ability to reassort.

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Introduction

Viruses in the family *Bunyaviridae* include some of the most important medical and agricultural pathogens. Members of the family are categorized into genera and species based on a combination of characteristics such as: vertebrate, arthropod and plant hosts; serological cross-reactivity to other members; and identity of protein sequences, particularly those of the nucleocapsid (N) protein (Fauquet et al., 2005). Currently 95 species in five vertebrate-, plant- and arthropod-infecting genera (*Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus*) are recognized (ICTV Master Species list v7, available at http://talk.ictvonline.org/files/ictv_documents/m/msl/1231.aspx). A feature common to the family is the presence of three genomic RNAs [termed small (S), medium (M) and large (L)] which encode the N protein, two glycoproteins (G_N and G_C) and an RNA-dependent RNA polymerase (L) [reviewed by Elliott (1997), Schmaljohn and Hooper (2001) and Nichol (2001)]. Two nonstructural proteins, NSs and NSm, are encoded on the S and M RNA, respectively, of the orthobunyaviruses, phleboviruses and tospoviruses. In the tospoviruses, both the M and S RNAs utilize an ambisense strategy with the non-structural proteins encoded in the viral sense and the structural proteins encoded in the viral complementary sense. For viruses in the genus *Tospovirus*, NSs has been shown to function as a suppressor of silencing (Takeda et al., 2002; Schnettler et al., 2010) and NSm has been shown to function as a movement protein (Lewandowski and Adkins, 2005; Li et al., 2009).

Apart from the hantaviruses, for which arthropod vectors are not known, all other viruses in the family *Bunyaviridae* are vectored by one or more arthropod species. Tospoviruses are transmitted by thrips, which must acquire the virus as larvae to become transmitters as adults (Sakimura, 1962; Wijkamp and Peters, 1993), whereas the other genera are transmitted by mosquitoes, phlebotomine sandflies, culicoid flies or ticks that feed on vertebrates (reviewed by Nichol, 2001).

The exchange of genetic material between viruses can occur in nature during cellular co-infections by two or more virus lineages either by recombination or reassortment. Such genetic exchange is presumably an underlying reason for the existence of segmented viral genomes which allows unique or novel combinations of distinct mutations to be combined, while undesirable changes are removed from the gene pool (Pringle, 1996). The creation of chimeric nucleic acid molecules derived from segments of each parental donor, termed recombination, is one mechanism for this type of exchange. However,

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to the best of our knowledge, recombination within the family *Bunyaviridae* has only been observed in the genus *Hantavirus* (Sibold et al., 1999). Reassortment is the exchange of complete genome segments in multisegmented viruses, and is another mechanism for genetic exchange. Reassortment of genomic RNAs has been reported within the vertebrate, plant or arthropod host for viruses of all genera in the *Bunyaviridae*, including orthobunyaviruses (e.g. Gentsch et al., 1980; Ushijima et al., 1981; Beatty et al., 1985; Borucki et al., 1999; Cheng et al., 1999; Briese et al., 2006; 2007), hantaviruses (e.g. Henderson et al., 1995; Rodriguez et al., 1998; McElroy et al., 2004), phleboviruses (e.g. Saluzzo and Smith, 1990; Turell et al., 1990; Sall et al., 1999), nairoviruses (e.g. Hewson et al., 2004) and tospoviruses (e.g. Best, 1961; Best and Gallus, 1955; Qiu et al., 1998). Studies of bunyavirus reassortment have proven useful because they have allowed mapping of attributes and functions to specific genomic RNA segments. For instance, encoding of the N protein by the S RNA was determined using reassortants (Gentsch et al., 1977) prior to the development of reverse genetics systems for bunyaviruses (e.g. Bridgen and Elliott, 1996; Flick and Pettersson, 2001; Billecocq et al., 2008).

Within the plant- and insect-infecting tospoviruses, indirect evidence of reassortment was first obtained from deliberate co-infection of plants with two strains of *Tomato spotted wilt virus* (TSWV) with subsequent observation of mixed phenotypic characters (Best and Gallus, 1955; Best, 1961). More recent direct evidence of reassortment has come from nucleic acid sequencing of local lesions derived from plants co-infected with two strains of either TSWV (Qiu et al., 1998) or *Watermelon silver mottle virus* (Okuda et al., 2003). The resulting isolates were identified as containing most, but not all, possible reassortment combinations. As with the vertebrate-infecting viruses, these reassorted tospovirus isolates facilitated the identification of regions associated with specific functions such as symptom determinants (Okuda et al., 2003). Additionally, a reassortant was observed to overcome transgenic host resistance (TSWV N gene-derived), a biological characteristic not present in either parental genotype (Qiu and Moyer, 1999).

Members of the genus *Tospovirus* collectively cause diseases in hundreds of plant species (Parrella et al., 2003) including many economically important crops plants such as tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*). *Groundnut ringspot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV) and TSWV are three genetically distinct species [based on N gene sequences (de Ávila et al., 1993)] but produce similar and often visually indistinguishable symptoms on tomato in South America (Gracia et al., 1999; Williams et al., 2001). GRSV was first isolated and described from both peanut (*Arachis hypogaea*) in South Africa and tomato in Brazil, whereas TCSV was first isolated from tomato in Brazil (de Ávila et al., 1990; 1993). Subsequently both GRSV and TCSV have been reported on tomato in Argentina (Dewey et al., 1995; Gracia et al., 1999; de Borbón et al., 2006; de Breuil et al., 2007). Other tomato-infecting tospoviruses, including Tomato zonate spot virus and Capsicum chlorosis virus, are only distantly related to TSWV, GRSV and TCSV (Dong et al., 2008; McMichael et al., 2002).

Based on serology and N gene sequence we recently identified GRSV from tomato plants with severe tospovirus symptoms in south Florida (Webster et al., 2010), a finding which extends the known distribution of this tospovirus beyond South America and South Africa (de Ávila et al., 1990, 1993) to North America. In the current study, we have determined and analyzed the full genome sequence of this virus. We demonstrate that the Florida GRSV isolate is actually an M RNA reassortant, with the L and S RNA segments indeed coming from GRSV but with the M RNA segment coming from TCSV to yield an $L_GM_TS_G$ genotype. We established that the $L_GM_TS_G$ genotype was widespread in tomato in south Florida and characterized some of its biological properties including vector transmission and plant resistance gene interactions. These data extend the current knowledge of the potential for reassortment within the family *Bunyaviridae*.

Results

Determination and analysis of $L_GM_TS_G$ genome sequence

The genome of a representative $L_GM_TS_G$ isolate collected in February 2010 from tomato in south Florida (Miami-Dade county) was completely sequenced. The genome was typical of a tospovirus with the three RNAs of 3067 nucleotides (nt) (S RNA, HQ644140), 4848 nt (M RNA, HQ644141) and 8876 nt (L RNA, HQ644142), and five predicted open reading frames (N = 777 nt, NSm = 912 nt, NSs = 1404 nt, G_NG_C = 3405 nt and L = 8625 nt). The conserved sequence motif 5′-AGAGCAAT-3′ or its reverse complement was present at the termini of each segment.

Nucleotide and amino acid (aa) comparisons of $L_GM_TS_G$ were made with previously sequenced and closely related tospoviruses (GRSV, TCSV, and TSWV). Comparisons of the $L_GM_TS_G$ N gene and deduced amino acid sequences with GRSV isolates in GenBank showed that identities were greater than or equal to 94.1% or 96.1% respectively, but were less than or equal to 83.3% or 88.0% at the nt or deduced aa level, respectively, with TCSV (Table 1). However, comparisons with the $L_GM_TS_G$ M RNA showed 97.6% and 91.7% nt identity to TCSV and GRSV, respectively, opposite to the trend observed with the N gene encoded on the S RNA. Similar values were seen across both coding regions (NSm and G_NG_C) of the M RNA. No comparisons to the L RNA of GRSV and TCSV could be made due to a lack of sequence information available in GenBank. Consistent low identity to TSWV (<80.7% nt and 89.6% aa; Table 1) was also seen for all three RNAs.

Sliding window analysis was also used to compare the level of nt identity between 200 nt segments of the M and S RNAs of $L_GM_TS_G$ and those of TCSV and GRSV isolates in GenBank (Fig. 1). The highest level of identity of the $L_GM_TS_G$ M RNA was observed with the TCSV M RNA across the entire NSm and G_NG_C coding regions in an individual window, as compared to GRSV and TSWV (Fig. 1A). Corresponding comparisons for the S RNA could not be made because of a lack of TCSV sequence data. However, a high level of identity (>94%) of the $L_GM_TS_G$ S RNA was observed with the GRSV S RNA (GenBank accession L12048; Dennis Gonsalves and Fuy-Jyh Jan, personal communication) across the entire NSs and N coding regions in an individual window, as compared to TSWV (Fig. 1B).

Table 1
Percentage identity of genomic RNAs and coding regions of $L_GM_TS_G$ with known isolates of GRSV, TCSV and TSWV.

Region	Identity with GRSV[a]	Identity with TCSV[a]	Identity with TSWV[a]
S RNA	**98.3% (1)**	–[b]	**76.1%**
N	**94.1–98.2% (4)**	**82.3–83.3% (2)**	**77.4%**
	96.1%–99.2% (4)	*84.5–88.0% (2)*	*79.4%*
NSs	**98.8% (1)**	–	**76.5%**
	99.8% (1)		*78.4%*
M RNA	**91.7% (1)**	**97.6% (1)**	**76.7%**
NSm	**91.8–93.5% (2)**	**98.1–98.2% (2)**	**80.7%**
	95.0–97.4% (2)	*99.3% (2)*	*86.1%*
G_NG_C	**91.6% (1)**	**97.6% (1)**	**76.0%**
	96.3% (1)	*98.4% (1)*	*81.3%*
L RNA	–	–	**77.3%**
L	–	–	**78.4%**
			89.6%

[a] Average percentage nucleotide (**bold**) and amino acid (*italics*) identity of $L_GM_TS_G$ to other full length tospovirus sequences in GenBank. *Groundnut ringspot virus* (GRSV): AF215271, AF213220, AF213673, AF513219, L12048, and S54327; *Tomato chlorotic spot virus* (TCSV): AF213674, AF282982, AF282983 and S54325; and *Tomato spotted wilt virus* (TSWV): NC_002051 (S RNA), AY744481 (M RNA) and AB198742 (L RNA). Percentages were determined in MEGA 4.1 with the numbers in parentheses indicating the number of sequences used for comparison.

[b] – = no sequence data available for comparison.

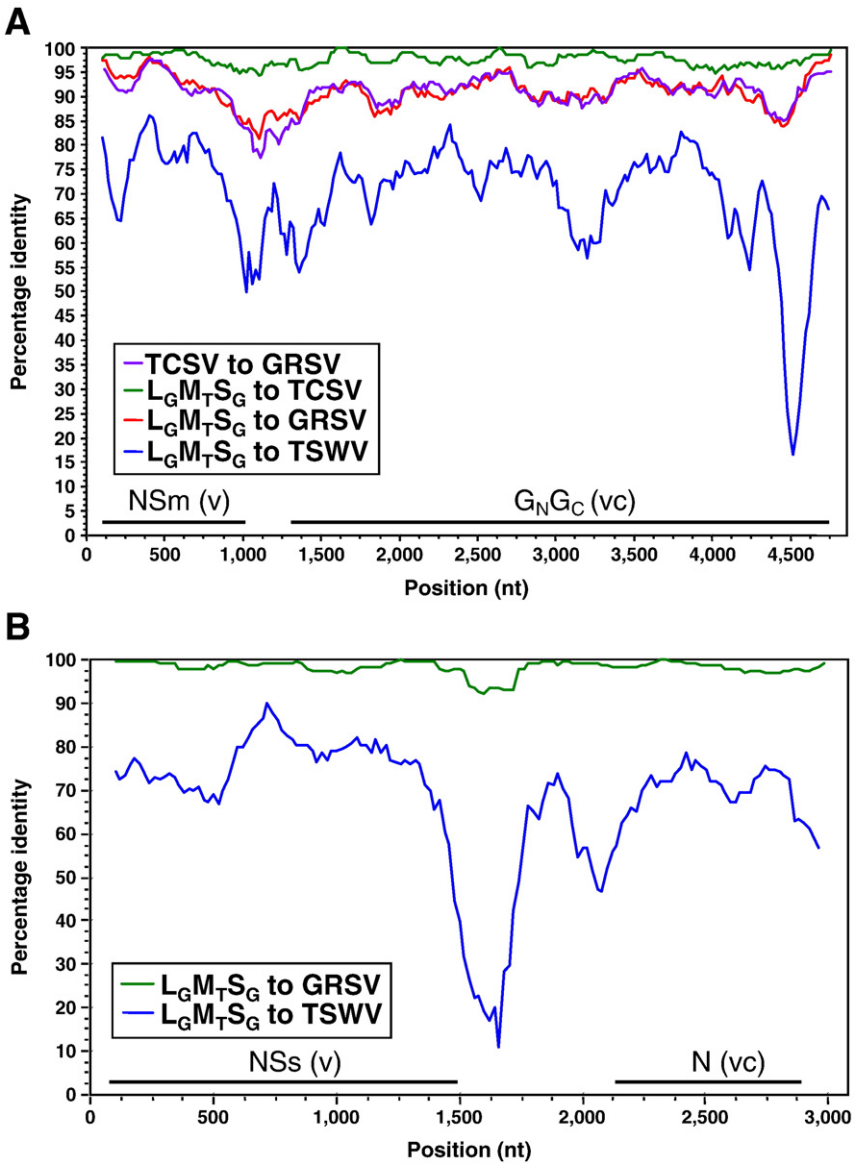

Fig. 1. Sliding window analysis of the genetic identity of $L_GM_TS_G$ M and S RNAs. A. Nucleotide identity of the M RNA of $L_GM_TS_G$ (HQ644141) and known isolates of *Groundnut ringspot virus* (GRSV, AF213673 and AY574055), *Tomato chlorotic spot virus* (TCSV, AF213674 and AY574054) and TSWV (AY744481). Single contigs of the 5′ and 3′ partial M RNA sequences of GRSV and TCSV were made. B. Nucleotide identity of the S RNA of $L_GM_TS_G$ (HQ644140) and known isolates of GRSV (L12048) and TSWV (NC002051). Genes and coding sense [viral (v) or viral complementary (vc)] are indicated at the bottom of each graph. SimPlot (Version 3.5) with a 200 nt window and a 20 nt step size was used.

Further evidence for the GRSV-TCSV reassortment origin of $L_GM_TS_G$ was obtained by cloning and sequencing portions of the N, NSm and L genes (providing representation of the S, M and L RNAs, respectively) from three known GRSV and two known TCSV isolates. Phylogenetic reconstructions were made for each of the genomic RNA regions sequenced of these and other GRSV and TCSV isolates available in GenBank (Fig. 2). The S and L RNAs of $L_GM_TS_G$ grouped most closely with all GRSV isolates, including the three known GRSV isolates sequenced as part of this study (Fig. 2A and C). The reverse was true for the M RNA with $L_GM_TS_G$ grouping closest to all available TCSV sequences (Fig. 2B). Both viruses also formed distinct clades with high bootstrap support for all three RNAs but the nucleotide diversity as measured by the substitution frequency per nucleotide (sub/nt from branch lengths in Fig. 2) was high for the S and L RNAs (>0.154 sub/nt for S RNA and >0.1696 sub/nt for the L RNA), whereas it was much lower for the M RNA (>0.0402 sub/nt). This indicates a much lower level of genetic diversity between these two species in the NSm and G_NG_C genes, than for the remaining genes of GRSV and TCSV.

Genetic homogeneity of $L_GM_TS_G$ isolates in Florida

Tomato plants (of multiple cultivars) with typical $L_GM_TS_G$ symptoms (generally more severe than TSWV symptoms in south Florida) including chlorotic and necrotic areas of leaves, and necrosis of petioles and stems, were collected from commercial fields in Miami-Dade, Hendry, Collier and Martin counties in south Florida. Short regions of sequence, 542, 670 or 644 nt each of the S, M and L RNAs, respectively, from 15 local lesion-passaged isolates (S, M and L RNAs) and 11 original field-collected samples (S RNA) showed very low nucleotide diversity (Table 2). The M RNA showed the most diversity with 3.7×10^{-3} sub/nt, whereas the S RNA showed 9.00×10^{-4} sub/nt (Table 2). These low diversity values

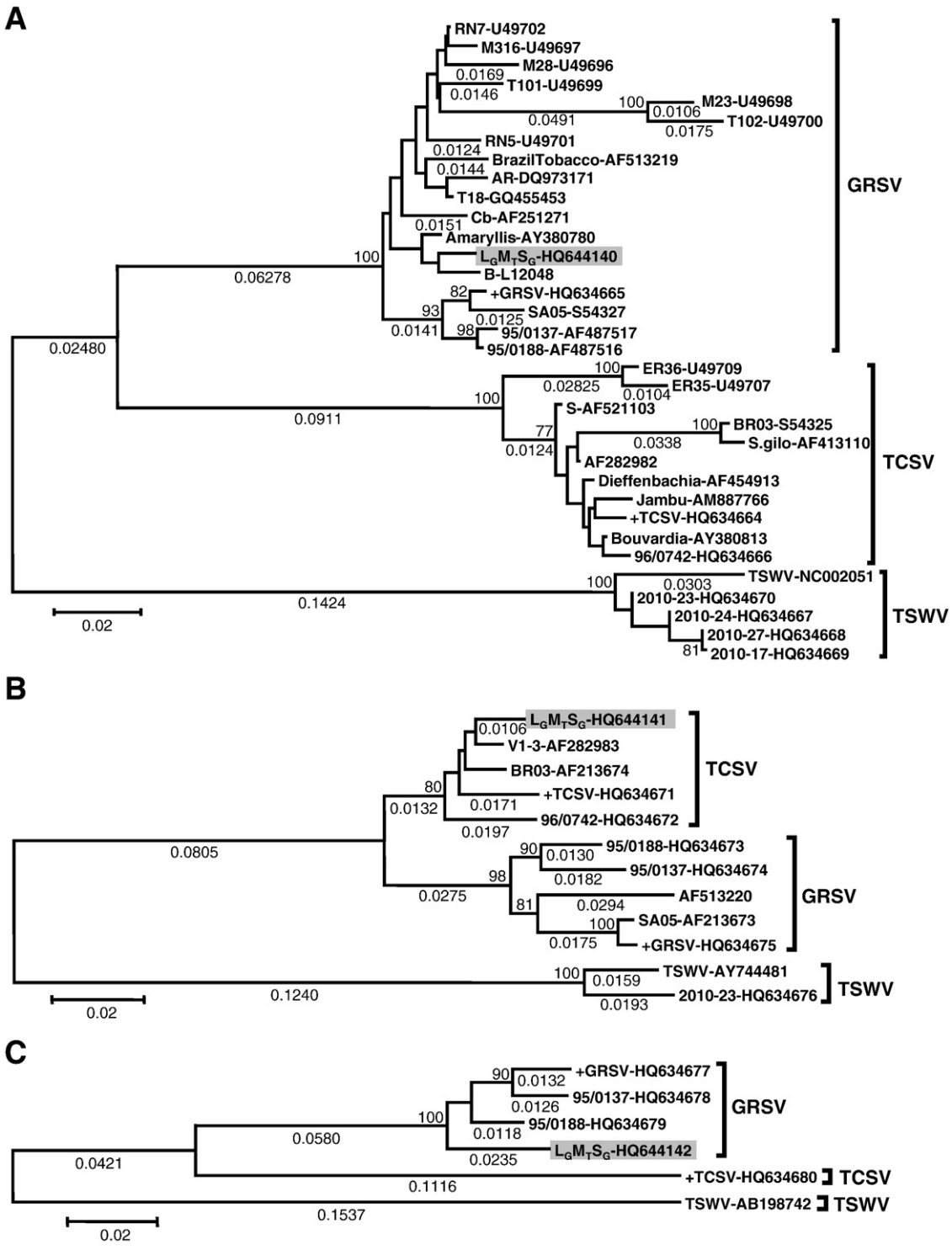

Fig. 2. Phylogenetic reconstruction of selected tospovirus species. A. S RNA (255–777 nt), B. M RNA (553–556 nt), and C. L RNA (628–633 nt) sequences. Both partial and full length sequences were aligned using Clustal W. Reconstructions were made using the neighbor-joining method in MEGA 4.1 with a maximum composite likelihood nucleotide substitution model with pairwise deletion. Bootstrapping (1000 replicates) was used to infer the robustness of the groupings with values over 70% indicated at nodes. Scale bar represents a genetic distance of 0.02 with distances greater than 0.01 included on the branches. Isolate designations and accession numbers of sequences from GenBank are included in taxon labels with $L_GM_TS_G$ highlighted in each tree.

indicate that a single population of $L_GM_TS_G$ is present in Florida and that the fully sequenced isolate is representative of the population.

No S or L RNA sequences of TCSV and no M RNA sequences of GRSV were detected by sequencing of more than 140 clones generated by RT-PCR using broad spectrum tospovirus primers from total RNA of both original field-collected samples and local lesion-passaged isolates. However, four tomato samples (2010–17, 2010–23, 2010–24 and 2010–27) infected with $L_GM_TS_G$ were also found to be infected with TSWV using specific primers (Fig. 2A and Fig. B).

Thrips transmission of $L_GM_TS_G$ isolates

A population of western flower thrips [*Frankliniella occidentalis* (Pergande)] originally collected from north Florida and known to

Table 2
Genetic diversity within $L_GM_TS_G$ isolates from Florida.

RNA[a]	S	M	L
π_{nt}[b]	9.00×10^{-4}	3.70×10^{-3}	3.57×10^{-3}
SE[c]	3.90×10^{-4} (26)	1.21×10^{-3} (15)	1.52×10^{-3} (15)

[a] 542, 670 or 644 nt segments of the S, M or L RNAs, respectively, were sequenced from 15 local lesion-passaged isolates (S, M and L RNAs) and 11 original field-collected samples (S RNA).
[b] Mean nucleotide diversity (π) of the pairwise sequence comparisons of each RNA segment was calculated in MEGA 4.1 using pairwise deletion and a maximum composite likelihood substitution model.
[c] Standard error (SE) of the mean with the number of isolates indicated in parentheses.

vector TSWV was used to determine whether this thrips species was also competent to acquire $L_GM_TS_G$ isolates from and transmit them back to tomato. Tomato plants (cv. Florida 47; Seminis Vegetable Seeds, Inc., St. Louis, MO) infected with one of eight local lesion-passaged $L_GM_TS_G$ isolates or three local lesion-passaged TSWV isolates (positive controls) were used as virus source plants with a total of five to 25 thrips per isolate (Table 3). Three mock-inoculated tomato plants were used as negative controls for virus acquisition by thrips. Leaf discs cut from uninfected tomato leaves were used as targets for transmission by thrips. Thrips and leaf discs were tested for virus by either ELISA or RT-PCR with equivalent results found by both methods.

Thrips were able to acquire all eight local lesion-passaged $L_GM_TS_G$ isolates (overall acquisition rate of 21.2%) and transmitted five of these isolates to tomato leaf discs with the overall transmission rate of 7.3%. Only 25 of 118 thrips tested positive for virus and were competent for transmission (Table 3). Thus, considering only leaf discs fed on by viruliferous thrips (i.e., those thrips that tested positive for $L_GM_TS_G$ virus acquisition), the overall transmission rate was 16.0%. Thrips were also able to acquire all three local lesion-passaged TSWV isolates (overall acquisition rate of 45%; about twice that of $L_GM_TS_G$) and transmitted all isolates to tomato leaf discs. Considering only leaf discs fed on by viruliferous thrips, the overall transmission rate of TSWV was 21.4% (similar to the 16.0% for $L_GM_TS_G$). Comparison of acquisition rates for the two viruses using a *G*-test of independence (Sokal and Rohlf, 1995) showed that the acquisition of TSWV was significantly greater than the acquisition of $L_GM_TS_G$ ($G=8.06$, df = 1, $P=0.0045$). Comparison of frequencies of virus-infected leaf discs among all leaf discs exposed to thrips, again using the *G*-test of independence, showed that thrips transmitted TSWV more efficiently than they transmitted $L_GM_TS_G$ ($G=15.31$, df = 1, $P<0.0001$). However, comparison of transmission frequencies for only viruliferous thrips, using the Fisher's exact test, showed that there was no difference in the transmission of $L_GM_TS_G$ and TSWV by viruliferous thrips ($P=0.69$). Thus, the apparent reduction in the transmission of $L_GM_TS_G$ seen in the non-adjusted data was due to a reduced ability of thrips to acquire $L_GM_TS_G$.

Virus was detected in six and ten additional leaf discs for $L_GM_TS_G$ and TSWV, respectively, despite no virus being detected in the corresponding thrips. Thrips from mock-inoculated plants did not become viruliferous although $L_GM_TS_G$ was detected in a single leaf disc fed on by these non-viruliferous thrips, presumably due to an escaped viruliferous thrips feeding on the disc.

The ability of thrips to transmit virus and cause systemic infection in intact Florida 47 tomato plants was also examined. Groups of adult thrips (reared as larvae on $L_GM_TS_G$-infected plants) were placed in clip cages on intact tomato plants for 48 h and then removed. Plants were monitored daily for symptoms of virus infection. Symptoms typical of $L_GM_TS_G$ infection in tomato (necrosis of leaf and petiole tissue) developed two weeks later. RT-PCR was used to confirm the presence of $L_GM_TS_G$ in each plant demonstrating that thrips are able to transmit $L_GM_TS_G$ to intact tomato plants and replicate the symptoms seen previously in tomato fields.

Table 3
Western flower thrips acquisition and transmission of $L_GM_TS_G$ and TSWV isolates.

Source plant isolate[a]	Virus acquisition[b]	Virus transmission[b]	Adjusted transmission[c]
$L_GM_TS_G$-A	4/23	3/31	1/4
$L_GM_TS_G$-B	2/8	1/9	0/2
$L_GM_TS_G$-C	1/8	0/12	0/1
$L_GM_TS_G$-D	4/25	0/25	0/4
$L_GM_TS_G$-E	2/5	0/6	0/2
$L_GM_TS_G$-F	6/14	1/17	1/6
$L_GM_TS_G$-G	4/19	2/25	1/4
$L_GM_TS_G$-H	2/16	3/21	1/2
Total $L_GM_TS_G$	21.2% (25/118)	7.3% (10/146)	16.0% (4/25)
TSWV-I	1/7	3/7	–[d]
TSWV-J	15/22	5/21	3/13
TSWV-K	2/11	5/13	0/1
Total TSWV	45.0% (18/40)	31.7% (13/41)	21.4% (3/14)
Mock-1	0/21	1/23	n/a
Mock-2	0/7	0/10	n/a
Mock-3	0/8	0/8	n/a
Total Mock	0.0% (0/36)	2.4% (1/41)	n/a (0/0)

[a] Eight local lesion-passaged $L_GM_TS_G$ isolates (A to H) and three local lesion-passaged *Tomato spotted wilt virus* (TSWV) isolates (I to K) were used to infect source tomato plants (Florida 47). Three mock-inoculated Florida 47 tomato plants (1 to 3) were used as negative controls.
[b] Determined by either ELISA using appropriate antibodies or RT-PCR using virus-specific primers and presented as the number of virus-positive thrips or leaf discs/number tested for each isolate. Numbers in parentheses indicate the total number of virus-positive thrips or leaf discs/total number of individuals tested for each source plant type.
[c] Transmission efficiency using results from viruliferous thrips only presented as the number of virus-positive leaf discs/number of leaf discs fed on by viruliferous thrips. Numbers in parentheses indicate the total number of virus-positive leaf discs/the number fed on by viruliferous thrips.
[d] Because of technical difficulties thrips and leaf disc sample sizes do not always correspond.

Responses of TSWV-resistant tomato and pepper cultivars to $L_GM_TS_G$

Twenty plants each of commercial tomato and pepper cultivars/lines with TSWV resistance were mechanically inoculated with a representative local lesion-passaged $L_GM_TS_G$ isolate. Comparisons could then be made of the response of the reassortant with reports of the parental (GRSV and TCSV) resistance reactions and to assess the potential of using such cultivars as part of a broader tospovirus management strategy that may be effective against both TSWV and $L_GM_TS_G$. TSWV-susceptible cultivars and a local lesion-passaged TSWV isolate (because we were unable to use GRSV and TCSV parental isolates) were included as controls.

TSWV resistance conferred by the *Sw5* gene in tomato cultivars Bella Rosa (Sakata Seed America, Morgan Hill, CA), BHN 602 and BHN 685 (BHN Seed, Immokalee, FL) was successful at deterring infection by both $L_GM_TS_G$ and TSWV (Table 4). Only one tomato plant (of 60) became infected with $L_GM_TS_G$ and none became infected with TSWV. $L_GM_TS_G$ infected 19 of 20 and TSWV infected 5 of 20 of the susceptible Florida 47 tomato plants inoculated with development of characteristic tospovirus symptoms.

In contrast, TSWV resistance conferred by the *Tsw* gene in an experimental pepper line was not successful in preventing infection by either $L_GM_TS_G$ or TSWV (Table 4). All pepper plants (20 of 20) became infected with $L_GM_TS_G$ and 9 of 20 were infected with TSWV. $L_GM_TS_G$ also infected 20 of 20 and TSWV infected 4 of 20 of the susceptible Aristotle (Seminis Vegetable Seeds, Inc., St. Louis, MO) pepper plants inoculated. Plants of both pepper types infected with either virus developed characteristic tospovirus ringspots and ring

Table 4
Response of TSWV-resistant tomato and pepper cultivars to $L_GM_TS_G$ and TSWV.

Plant	Cultivar	Resistance gene	$L_GM_TS_G$			TSWV		
			Symptoms[a]	Upper leaf[b]	Root[b]	Symptoms[a]	Upper leaf[b]	Root[b]
Tomato	Florida 47	–	C,N,LD	19/20	17/20	C,LD	3/20	5/20
	Bella Rosa	*Sw5*	NS	0/20	0/20	NS	0/20	0/20
	BHN 602	*Sw5*	NS	0/20	0/20	NS	0/20	0/20
	BHN 685	*Sw5*	C,N	1/20	0/20	NS	0/20	0/20
Pepper	Aristotle	–	C, RS, LD	20/20	16/20	RS, LD	2/20	4/20
	experimental	*Tsw*	C, RS, LD	20/20	19/20	RS, LD	8/20	9/20

[a] Symptoms of infection: C, chlorosis; N, stem and petiole necrosis; LD, leaf deformation; RS, ringspots/ring patterns; NS, no symptoms on inoculated leaves.
[b] Virus infection of upper, uninoculated leaves or roots determined by ELISA of each plant tested at two (pepper) or three (tomato) weeks post-inoculation.

patterns on inoculated leaves, and deformation and chlorosis of upper, uninoculated leaves.

Though not known at the outset, the TSWV isolate used as a control in these experiments appears to be a *Tsw* gene resistance breaking isolate. In fact, about twice as many of pepper plants containing the *Tsw* resistance gene were infected in comparison to the susceptible Aristotle pepper plants. Total RNA from three symptomatic *Tsw* gene-containing resistant pepper plants inoculated with TSWV was tested by RT-PCR for both viruses and this test confirmed that only TSWV (and not $L_GM_TS_G$, or a mixture of $L_GM_TS_G$ and TSWV) was present in these plants.

Discussion

Reassortment of genomic RNAs is one mechanism widely employed by multi-segmented viruses to adapt to a changing environment. Viruses in the five genera in the family *Bunyaviridae* are known to reassort genomic RNAs within a given species. Reassortment is often shown by experimental co-infection of a single host with two different strains (or isolates) of the same virus species. Reassortment can occur naturally (or experimentally) within a virus species, but has also been shown to occur between species although at an apparently lower frequency than reassortment within a species (e.g. Rodriguez et al., 1998; Briese et al., 2007). However, reassortment has not previously been reported between species in the plant- and insect-infecting genus *Tospovirus*.

In this report, we provide the first demonstration of a natural reassortant between GRSV and TCSV, two species in the genus *Tospovirus*. Upon detailed examination, what we first identified as GRSV (Webster et al., 2010) was instead discovered to be a stable and widespread M RNA reassortant arising from GRSV and TCSV. This reassortant was confirmed to have an $L_GM_TS_G$ genotype by nucleotide sequence comparisons to known GRSV and TCSV isolates from around the world (Table 1, Fig. 2). No evidence for recombination (another widespread mechanism for introducing genome variation) between GRSV and TCSV was detected in $L_GM_TS_G$ by sliding window analysis of full length M RNA genomic segments (Fig. 1). Nucleotide and amino acid identities of the full length $L_GM_TS_G$ sequence were over 97.6% with the sequences of each parental genomic segment indicating each RNA likely was derived from the indicated species (Table 1). Overall low genetic diversity was seen in all isolates collected from Florida in all the genomic regions analyzed (Table 2).

In South America, where GRSV, TCSV and TSWV all occur, co-infection of two species, which is required for reassortment to occur, has not been reported in recent descriptions (Williams et al., 2001; de Borbón et al., 2006; de Breuil et al., 2007). Instead, geographic separation of all three species, particularly within Argentina, has been observed (Gracia et al., 1999; Williams et al., 2001). We note, however, that four tomato samples analyzed in the present study were found to be co-infected with $L_GM_TS_G$ and TSWV. Most studies with tospovirus genetics have focused on the plant host but it should also be considered that reassortment may occur in the thrips vector, as shown for the insect vectors of vertebrate-infecting members of the family *Bunyaviridae* (Beatty et al., 1985; Turell et al., 1990).

$L_GM_TS_G$ was the only reassortant genotype recovered in Florida despite our testing of multiple local lesions derived from field samples collected from different locations and at different times of the year — including local lesions from the four tomato samples with $L_GM_TS_G$ and TSWV co-infections. Our approach of using broad-spectrum tospovirus primers, and cloning and sequencing the resulting amplicons makes it improbable that undetected mixed infections of GRSV and TCSV occurred in Florida. It is therefore likely that $L_GM_TS_G$ was introduced to Florida from overseas in its present form in the relatively recent past. This scenario would also explain why neither of the two parental genotypes (GRSV or TCSV) was found.

Several tospovirus phenotypes (biological characteristics) have been linked to specific genomic RNA segments (and thus genotypes). Consequently, we explored two of these biological characteristics (acquisition and transmission by western flower thrips, and reaction to TSWV resistance genes) for $L_GM_TS_G$ for comparison to reports for GRSV and TCSV. Western flower thrips transmitted $L_GM_TS_G$ at a lower rate than TSWV (Table 3), consistent with previous results showing less efficient transmission of GRSV and TCSV by western flower thrips (Wijkamp et al., 1995). The picture is complicated by the facts that the virus isolate (de Borbón et al., 2006), the thrips population (Wijkamp et al., 1995) and repeated mechanical passaging (e.g. Nagata et al., 2000; Resende et al., 1991) can affect transmission efficiency. Therefore, it is perhaps unsurprising to find a report of the equal efficiency of TCSV and TSWV transmissibility (Nagata et al., 2004). The genetic determinants of TSWV transmission by thrips have been mapped to the M RNA (Sin et al., 2005), so an M RNA reassortant like $L_GM_TS_G$ might well be expected to show transmissibility more similar to TCSV than to GRSV. But as noted above, western flower thrips transmission of TCSV and GRSV isolates may both be low compared with transmission of TSWV (Wijkamp et al., 1995; de Borbón et al., 2006) and we observed no clear difference between $L_GM_TS_G$ and reports for GRSV and TCSV. In spite of this outcome, our results with $L_GM_TS_G$ demonstrating a reduced acquisition efficiency (21.2% vs. 45.0% in TSWV) but equivalent transmission efficiency (16.0% vs. 21.4% in TSWV, Table 3) may offer some novel insight into the relative transmission efficiency previously reported for GRSV and TCSV compared to TSWV. Likely the reduced acquisition of $L_GM_TS_G$, GRSV and TCSV by western flower thrips is due to a less efficient transit of these virions across membrane barriers in the thrips (Whitfield et al., 2005) in comparison to TSWV. Transmission of $L_GM_TS_G$ by thrips species present in south Florida is apparently sufficient for this reassortant to have become established in four counties even though only 7.3% of the total leaf discs in our experiment became infected with $L_GM_TS_G$ (Table 3). Further investigations are ongoing into the role that other thrips species, including *Frankliniella schultzei* (Trybom), which is reported to be a more efficient vector of GRSV (Wijkamp et al., 1995; Nagata et al., 2004) and has been found in proximity to tomatoes infected with $L_GM_TS_G$ isolates (Webster et al., 2010), may play in the disease cycle of $L_GM_TS_G$ in Florida.

Mirroring the similarity observed between the reassortant and parental genotypes regarding the transmission by western flower thrips, the interaction of $L_GM_TS_G$ with the TSWV *Sw5* and *Tsw* resistance genes in tomato and pepper, respectively, did not differ from the reported interaction of the parental genotypes with these genes. Specifically, the *Sw5* gene in tomato conferred resistance to $L_GM_TS_G$, whereas the *Tsw* gene in pepper was overcome, as previously reported for GRSV (Table 4; Boitreux and de Ávila, 1994). The inability of $L_GM_TS_G$ to overcome *Sw5* resistance was also unsurprising, as previously *Sw5* resistance breaking was mapped to the M RNA of TSWV (Hoffmann et al., 2001), and GRSV and TCSV are unable to overcome this resistance (Boitreux et al., 1993; Boitreux and Giordano, 1993). In the course of these experiments, we also discovered that the isolate of TSWV that we used as a control is able to overcome *Tsw* but not *Sw5* resistance, which is consistent with previous findings of resistance breaking by some TSWV isolates collected from the field (Roggero et al., 2002). While the infectivity of this isolate was also reduced compared to $L_GM_TS_G$, we confirmed that the symptoms we observed in "resistant" peppers were due solely to infection by TSWV.

The close genetic relatedness of the M RNA segments of GRSV and TCSV (Fig. 1; Silva et al., 2001; Lovato et al., 2004) may suggest a higher negative selection being imposed on G_NG_C and NSm sequences. Alternatively, this may be evidence of a more distant reassortment event between TCSV and GRSV because of the high nucleotide diversity in the S and L RNAs of the two species but relatively low diversity of the M RNA (Fig. 2). The lack to date of non-reassorted parental genotypes in Florida makes this hypothesis difficult to prove unless a virus is found with a significantly different M RNA. The factors determining the success of reassortant progeny have not been fully explained and while a close genetic relationship between parental genomes seems to be required other factors also appear important. The evidence of non-random generation of reassortants (Urquidi and Bishop, 1992; Qiu et al., 1998) and relative ease of formation of M RNA reassortants (Briese et al., 2007; Rodriguez et al., 1998) indicate that there are likely unexplored interactions between the genes encoded by the L and S RNAs. This may suggest a relatively independent role for the G_N, G_C and NSm proteins (encoded by the M RNA) with few interactions to the L, NSs or N proteins (encoded by the L and S RNAs).

There exists a tradeoff between the likelihood of success of reassorted progeny and the potential rewards of bringing together diverse gene combinations. The occurrence and frequency of genomic RNA reassortment between bunyaviruses are closely linked to the degree of sequence relatedness between the parental viruses (Bishop, 1985), the reasons for which likely include the need for progeny viruses to have complementary genes from the different genomic RNA segments (Garcia-Arenal et al., 2001). Isolates of a single bunyavirus species (such as *California encephalitis virus*) show the highest potential for reassortment (Gentsch and Bishop, 1976; Gentsch et al., 1977) although reassortment can also occur between distinct species (this study; Briese et al., 2007; Rodriguez et al., 1998). Insufficient information is available to accurately define a limit of genetic diversity between genomic RNAs that can successfully reassort but it does appear to be less than the diversity found between the serogroups in the family *Bunyaviridae* because to date no natural or artificial reassortments have been described from separate serogroups. It has been proposed that viruses which are able to exchange genome segments and create viable reassortants should be considered a single species (VanRegenmortel et al., 1997). This simple distinction, however, is perhaps inadequate with reassortants within the *Bunyaviridae* being described between species by both artificial (Rodriguez et al., 1998; Kang et al., 2002; McElroy et al., 2004) and natural (Briese et al., 2007) methods and would result in the collapse of many currently accepted species down to just a few. Such a reduction in species would not accurately represent the genetic and biological differences observed in this diverse virus family.

Our identification of an interspecific tospovirus M RNA reassortant highlights some of these issues facing taxonomy in the family *Bunyaviridae*. It provides further evidence that identification of a tospovirus species using only N gene sequences may not be sufficient, and supports the suggestion of others (e.g. Tspompana et al., 2005) that sequence data from all three genomic RNA segments are needed to definitively confirm species identification. Current guidelines for the inclusion of newly identified viruses as species in the family are based on combinations of biological data (host and vector species), serological data (cross-neutralizing and cross-hemagglutination inhibition tests) and sequence data (amino acid identity of the N or G_NG_C proteins), the specifics of which vary between genera (Fauquet et al., 2005). Demarcation of species based solely on sequence identity will always be problematic due to the processes of reassortment and recombination, which occasionally can occur between species and often creates novel phenotypes.

The taxonomic community may be able to address limitations with the current taxonomy of *Bunyaviridae* species demarcations with respect to interspecific reassortants by considering naturally occurring reassortants that persist in the environment in one of two ways: 1) where reassorted viruses show high amino acid identity to the segments of all parental viruses and have similar biological data (as with $L_GM_TS_G$) that they be regarded as strains of the parental virus contributing the majority of the genomic RNA segments and 2) where reassorted viruses demonstrate biological differences such as host specificity or pathogenicity, then they should be regarded as distinct species even if amino acid identity of genomic RNA segments with parental species is above accepted species demarcation limits. By this strategy, $L_GM_TS_G$ should be regarded as a strain of GRSV due to the high nucleotide identity shared with the GRSV L and S RNAs, the similar western flower thrips transmission parameters and the interactions with TSWV resistance genes.

Materials and methods

Isolation of tospoviruses

Tomato samples with typical $L_GM_TS_G$ symptoms described above were collected from commercial tomato fields in Miami-Dade, Collier, Hendry and Martin counties in south Florida between December 2009 and June 2010. Local lesions were generated by mechanical inoculation of *Nicotiana glutinosa* or *Nicotiana benthamiana* plants with tomato tissue homogenized in 0.5% (w/v) sodium sulfite. Two to three separate local lesions were chosen from each isolate and used to independently inoculate systemic tospovirus hosts including American black nightshade (*Solanum americanum*), jimsonweed (*Datura stramonium*) and tomato (*Solanum lycopersicum* cv. Florida 47). Following the development of systemic symptoms in these hosts, individual isolates were propagated by mechanical and/or graft inoculation in additional plants of the same species. All plants were grown and maintained in a greenhouse. A local lesion-passaged TSWV isolate generated from a tomato plant collected in Indian River county in 2009 was used as a control throughout these experiments.

RT-PCR and serological detection of tospoviruses

RT-PCR and enzyme-linked immunosorbent assays (ELISA) were used for the detection of tospoviruses in plant material and thrips as described previously (Webster et al., 2010). Briefly, Trizol Reagent (Invitrogen, Carlsbad, CA) extracted total plant RNA or total thrips nucleic acid (13 µl used immediately for cDNA synthesis; Rotenberg et al., 2009) was used as template for RT-PCR (MMLV and GoTaq; Promega Corp., Madison, WI) to amplify $L_GM_TS_G$ and TSWV N gene fragments. Virus specific primers for the N gene of GRSV (GRSV-N-v/GRSV-N-vc, Table 5) and TSWV (722/723; Adkins and Rosskopf, 2002) gave amplicons of the expected size (GRSV 594 nt, TSWV 620 nt) that were excised from 1.0% agarose gels, purified (QiaQuick gel purification kit; Qiagen Sciences, Germantown, MD) and directly

Table 5
Sequences of primers for $L_GM_TS_G$ genome sequencing and isolate genotyping.

Name	Sequence (5′ to 3′)	Genome location[a]
GRSV-N-v	AGAGCTTCCTTAGTGTTGTACTTAG	N
GRSV-N-vc	GAAAGGTCTAGATCTAAACTGCCAC	N
GRSV-NSs-v	TAAGCACAAGAGCACAAGAGCCAC	NSs
GRSV-NSs-vc	GCCTGATTGGTGGCTCATTTCGAC	NSs
TospoSgap-v	CTGATTCTCATTTTAAGTTGAGCC	IGR S RNA
TospoSgap-vc	CCCTCTCATTGCTTCTGTTGACAG	IGR S RNA
Sgap2-v	GTGGCTCATACTGCTTCAAGTAG	IGR S RNA
Sgap2-vc	CGTGTTAAGTGTGATAAAGTCAGG	IGR S RNA
GLY1-v	TGGACGAGATATAGTATCATGCTG	G_NG_C
GLY1vc	TATGGAATCTTGTTCTCTATCTAGC	G_NG_C
GLY2-v	CTTCTTATCTGGATTAACCGAGC	G_NG_C
GLY2-vc	TACGATTTATATAAGGAGCACAGG	G_NG_C
GLY3-v	GCAGTAATAATCTGTGTGAGTGC	G_NG_C
GLY3-vc	CATCTACAAACAAACAGCTTGCATC	G_NG_C
GLY4-v	CAGAAGCTTAGTAGTCTTATTCA	G_NG_C
GLY4-vc	AGCATTCTGTTGTTCAGGGCTAC	G_NG_C
GLY5-v	CAGAGTGTGCTTTGAAGTTCCC	G_NG_C
Mgap-v	CTGAACGTAGCAAGTCCACAAG	IGR M RNA
Mgap-vc	GTTAGACGGAAATCATAATCTC	IGR M RNA
3′Lgap-v	CGGATAGTGGCAAGAACCCAGC	L
3′Lgap-vc	CGGCATCATTAAGTAATTCAG	L
5′Lgap-vc	GGCTGTGGAACATGGGTCTG	L
3RdRp-v	TTCCAGGCAGGTGTCTTTGATG	L
5RdRp-vc	GGTATATTGCCAAGTCGTTACAC	L
$L_GM_TS_G$ 1L-v	GTTAGCGAATTCATCAACCCG	L
$L_GM_TS_G$ 1L-vc	TAGCAATAAACACCTGCTTCTCC	L
$L_GM_TS_G$ 2L-v	GTCCAATTTGTAATTAGCTGTGG	L
$L_GM_TS_G$ 2L-vc	TTCAAAGAATTAGAGGAAAAGCAC	L
$L_GM_TS_G$ 3L-v	CGCAGCTTAGTTCTGTCAAGC	L
$L_GM_TS_G$ 3L-vc	GAAGCCTATGGGAGGAATGCC	L
$L_GM_TS_G$ mid2-v	TTGAGGTAACTTAATGATCTGTG	L

[a] IGR = intergenic region.

sequenced (as described below). Commercially available ELISA reagents for TSWV and GRSV/TCSV (Agdia Inc., Elkhart, IN) were used according to the manufacturer's instructions.

Sequence and analysis of $L_GM_TS_G$ genome

An $L_GM_TS_G$ isolate originally identified in tomato from Homestead, FL (Miami-Dade county) in February 2010 and purified by two successive local lesion passages (as described above), was selected for genome sequencing. Amplicons generated by RT-PCR using primers that anneal to conserved tospovirus sequences (Cortez et al., 2001; Silva et al., 2001; Chu et al., 2001) were ligated into pGEM-T (Promega). Additional primers (Table 5) for determining the complete $L_GM_TS_G$ genome by a genome walking strategy were designed from partial $L_GM_TS_G$ sequences and available tospovirus sequences (GenBank accessions AF213673, AF213674, AF251271, AF282983, AF487516, AF487517, AF513219, AF513220, AY574054, AY574055, L12048 and S54327).

Total nucleic acids from plants infected with three known GRSV isolates and two known TCSV isolates were kindly provided by Dr. Francisco Assis (Agdia, Inc., Elkhart, IN) and Dr. Neil Boonham (Food and Environmental Research Agency, United Kingdom). Selected portions of the L, M and S RNAs of these isolates were amplified and cloned for comparison to the corresponding regions of the $L_GM_TS_G$ genome.

Recombinant plasmids and PCR amplicons were sequenced in both directions on an ABI3730XL automated sequencer at the USHRL Sequencing Support Laboratory. Vector NTI Advance software (Version 11, Invitrogen, Carlsbad, CA) was used for consensus construction of the $L_GM_TS_G$ genome and known GRSV and TCSV isolate sequences.

MEGA (Version 4.1, Tamura et al., 2007) with Clustal W and default parameters was used for sequence analysis and alignment. Nucleotide and amino acid identities were calculated from pairwise comparisons of the number of substitutions using the pairwise deletion option and the number of common sites. Neighbor-joining trees were constructed using 1000 bootstraps to infer the robustness of the groups. Sliding window comparisons of nucleotide sequences were made using SimPlot (Version 3.5, Lole et al., 1999) using a 200 nt window and step size of 20 nt.

Thrips maintenance and tospovirus acquisition and transmission

A colony of western flower thrips maintained on green bean (*Phaseolus vulgaris*) pods and known to vector TSWV was used to determine whether this thrips species was also competent for $L_GM_TS_G$ transmission to tomato leaf discs as previously described (Wijkamp et al., 1996). Briefly, recently emerged first instar larvae (<12 h old) were placed onto cuttings from Florida 47 tomato plants infected with $L_GM_TS_G$ or TSWV (positive control), or mock-inoculated (negative control). Following a 48 h acquisition access period, thrips larvae were moved to fresh green bean pods and reared to adulthood (ten days). Individual adult thrips were transferred to leaf discs prepared from uninfected Florida 47 tomato plants with a number 10 cork borer, 14 mm diameter, and allowed to feed for 48 h. Thrips were then collected and stored at −20 °C for later analysis, and leaf discs were floated on distilled water for five days to allow replication of any transmitted virions to levels detectable by RT-PCR and ELISA. A minimum of three thrips and leaf discs were tested by RT-PCR for each local lesion population, while ELISA was used for the remainder due to time constraints. No significant difference in results between the testing methods was observed. The *G*-test of independence was used to determine whether the frequency of acquisition and transmission differed between $L_GM_TS_G$ and TSWV, and the Fisher's exact test was used to determine whether the frequency of transmission by viruliferous thrips differed between $L_GM_TS_G$ and TSWV (Sokal and Rohlf, 1995).

Groups of two, eight or eleven adult thrips (reared as larvae on $L_GM_TS_G$-infected tomato as described above) were also transferred to clip cages, placed on intact Florida 47 tomato plants and allowed to feed for 48 h. Thrips were then removed and plants were returned to the greenhouse and monitored daily for symptoms.

Inoculation of TSWV-resistant tomato and pepper cultivars

Twenty plants each of TSWV-resistant and susceptible tomato and pepper cultivars/lines were mechanically inoculated with homogenized leaf tissue (as described above) infected with either a representative local lesion-passaged $L_GM_TS_G$ or the local lesion-passaged TSWV isolate (described above). $L_GM_TS_G$- and TSWV-inoculated plants were maintained in separate, air-conditioned greenhouses and monitored daily for symptoms.

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